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13. ABSTRACT (Maximum 200 Words)
The structure and homestasis of normal breast parenchyma is maintained by dynamic interactions between breast epithelial cells and their associated stroma. During breast carcinogenesis, these stromal-epithelial interactions are increasingly deregulated. Stromal fibroblasts in invasive breast carcinomas (i.e., carcinomas associated fibroblasts, CAF) differ from fibroblasts associated with normal breast. These differences include the production of increased amounts of type-specific collagens, the over expression of various growth factors, proteases and protease inhibitors, and acquisition of the myofibroblast phenotype, characterized by alpha-smooth muscle actin (SMA) expression. SMA functions to stop the migraton of breast fibroblasts and contributes to the contraction of myofibroblasts. These activities involve alterations in adhesion moleculares and cytoskeletal organization, which also affect expression of other molecules, such as extracellular matrix (ECM) proteins and proteases, by fibroblasts. In this project we test the hypothesis that expression of SMA is responsible for much of the CAF phenotype. RNA interference was utilized to inhibit expression of SMA in CAF. SMA-inhibited and SMA-expressing CAF were compared for the expression of a variety of cell adhesion molecules, ECM proteins, and ECM modulating proteases. We demonstrate that SMA in CAF affects the expression of several cell adhesion molecules, ECM proteins and ECM

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Table of Contents

INTRODUCTION:

The structure and homeostasis of normal breast parenchyma is maintained by dynamic interactions between breast epithelial cells and their associated stroma. During carcinogenesis, these stromal-epithelial cell interactions are increasingly deregulated. In invasive breast carcinomas, the surrounding stroma is characterized by populations of fibroblasts, which differ from fibroblasts associated with normal breast. These differences include the production of increased amounts of type-specific collagens, the over expression of various growth factors, proteases and protease inhibitors, and acquisition of the myofibroblast phenotype, characterized by alpha-smooth muscle actin (SMA) expression [1]. *In vitro* and *in vivo* evidence indicates that carcinoma-associated fibroblasts (CAF) can act to enhance the progression of breast carcinoma [2-7]. SMA has been considered primarily as a marker of the myofibroblast phenotype, but it also has a functional effect. It stops the migration and slows the proliferation of breast fibroblasts, and contributes to the contraction of myofibroblasts during wound healing [8, 9]. These activities involve alterations in adhesion molecules and cytoskeletal organization [8-10], which also influences the expression of other molecules, such as extracellular matrix (ECM) proteins and proteases, by fibroblasts. These SMA-induced changes in the expression pattern of fibroblasts are likely to affect the interactions of fibroblasts with adjacent epithelial cells. In this research, we begin the investigation of SMA as a molecular target to alter the phenotype of fibroblasts and thereby modulate fibroblast-epithelial cell interactions involved in cancer progression. Specifically, we utilize RNA interference to suppress expression of SMA in CAF and assess the effect on expression of ECM proteins, cell adhesion molecules, growth factors and related molecules. The result of SMA inhibition in CAF on fibroblast-epithelial cell interactions that impact proliferation and invasiveness of epithelial cells will be evaluated.

BODY:

TASK 1 -   To characterize the effect of SMA on the expression profile of CAF (Months 1 - 9).

a. Isolate cancer associated fibroblasts (CAF) and fibroblasts associated with histologically normal breast (NAF) from 10 breast cancer resection specimens

We have isolated CAF from 11 human breast cancer specimens, and NAF from 11 human breast reduction specimens and from histologically normal breast in 5 breast cancer resection specimens. The fibroblasts were isolated using a standard dissociation protocol with a differential trypsinization at the first passage to eliminate epithelial cell contamination. A portion of the tissue sample from which the fibroblasts was isolated was examined histologically by frozen sectioning and staining with hematoxylin and eosin to confirm the presence or absence of cancer as indicated. The fibroblasts have been characterized and confirmed to be fibroblasts by immunocytochemistry for fibroblast-related markers, such as vimentin, and epithelial-related markers, such as epithelial membrane antigen and cytokeratins. We have also begun to compare expression of selected molecules, including SMA, in CAF versus NAF.

In vivo, SMA is expressed in CAF and fibroblasts around DCIS at a much higher level than in normal breast. To compare the level of expression of SMA in CAF and NAF *in vitro*, 5 CAF cultures and 3 NAF cultures were grown on coverslips in DMEM supplemented with 10% fetal bovine serum (FBS). The presence of SMA was detected by immunocytochemical staining with anti-SMA (mouse monoclonal antibody, clone 1A4, DakoCytomation) and the percentage of positively staining cells assessed. The average percent positive cells in both CAF and NAF when grown on coverslips is relatively high (58% and 59%, respectively) and similar in the two types of fibroblasts (Figure 1). This is not a surprising finding since an increase in SMA expression by fibroblasts when grown on rigid substances, such as cover glass or plastic culture dishes, in the presence of serum, is well-known [9]. These conditions provide the mechanical stress and the TGF-β required for myofibroblastic differentiation. However, when fibroblasts are grown as three dimensional (3D) aggregates on ECM (i.e., Matrigel), the

4

expression of SMA in CAF and NAF differs. 2 CAF cultures and 2 NAF cultures were grown in 3D-aggregates on Matrigel with serum-containing medium. The cultures were fixed in formalin and processed to paraffin. Histologic sections of the 3D cultures were immunostained with anti-SMA and the percentage of positively staining cells assessed (Figure 1). In these conditions, 2% of NAF and 50% of CAF expressed SMA. The expression of SMA in CAF and NAF in 3D growth on ECM more closely resembles the expression pattern observed *in vivo*. This likely is the result of two factors. 1) Growth of fibroblasts in 3D aggregate results in a growth arrested state and is considered to more closely resemble the *in vivo* state than standard culture conditions [11]. 2) ECM is a relatively low stiffness substrate and provides less mechanical stress than plastic or glass, but when attached, at least partially, to the culture vessel provides enough mechanical stress to allow expression of some SMA [9]. These results were also confirmed by Western blot analysis for SMA in one NAF and one CAF culture grown on plastic and on Matrigel. Based on these findings, we plan to grow NAF and CAF, expressing SMA and SMA-inhibited, in 3D cultures prior to RNA isolation and gene expression analysis to more closely simulate *in vivo* growth.
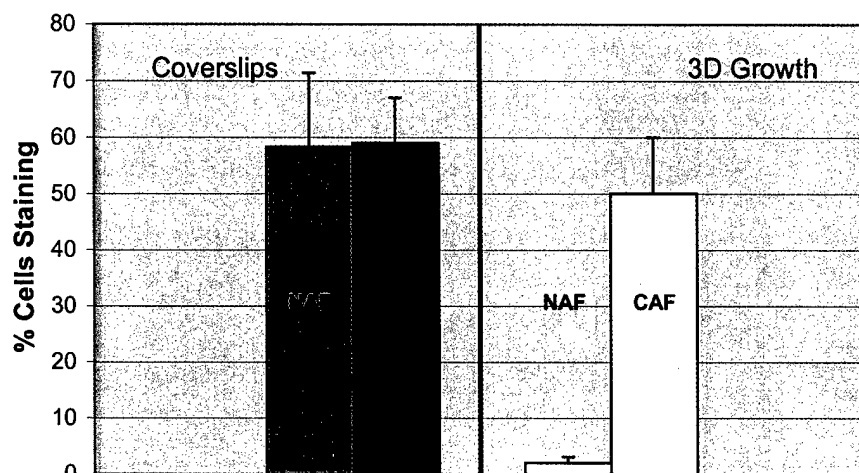

Figure 1. SMA expression in breast fibroblasts grown on glass coverslips or as 3D aggregates on ECM. The percentage of cells expressing SMA, as detected by immunocytochemistry in NAF and CAF grown on coverslips in serum-containing medium is similar. However, when NAF and CAF are grown as 3D aggregates in EMC (Matrigel) the percentage of cells staining positively with anti-SMA is lower in NAF than CAF. Thus, the 3D culture conditions more closely simulate SMA expression *in vivo*.

b. Transfect each CAF with siRNA to interfere in expression of SMA

To further our understanding of the role of SMA in the CAF phenotype, we inhibited the expression of SMA protein using RNA interference. RNA interference is a method of post-transcriptional gene silencing mediated by small (21-23 nucleotides), interfering RNAs (siRNAs)[12]. Double stranded siRNA with sequence specificity for SMA was designed using the Oligoengine siRNA Design Tool (www.oligoengine.com). Multiple potentially effective siRNA sequences were generated. Two were selected for use based on an absence of homology with other human genes, particularly other actin isoforms, determined by a BLAST (www.ncbi.nlm.nih.gov/blast) search. Additionally, a control siRNA consisting of the same base composition as one of the selected siRNAs, but scrambled, was designed. Again a BLAST search was performed to assure an absence of homology of the control

siRNA with other human genes, particularly the actin isoforms. The siRNAs were synthesized by Dharmacon RNA Technologies (www.dharmacon.com) and transfection of CAF grown on plastic was achieved using the Mirus *Trans* IT-TKO transfection reagent, as per the manufacturer's instructions. Transfection conditions and quantity of siRNA utilized were optimized to achieve the highest level of suppression of SMA expression with the lowest level of toxicity to fibroblasts. Under these conditions, Western blot analysis demonstrated minimal detectable SMA in the CAFs transfected with SMA-specific siRNAs, relatively strong expression of SMA in the control transfectants, and weaker expression of SMA in non-transfected control CAF (Figure 2). To quantify the degree of inhibition of SMA, the expression level was measured by densitometry, the background was subtracted, and the expression of the SMA-specific siRNAs was divided by the control siRNA. This resulted in an inhibition of SMA protein expression of 80 - 95%. Complete elimination of SMA expression would be ideal. However, the level of SMA suppression achieved should be sufficient to demonstrate the effect of SMA on the CAF phenotype, and may be appropriate since a low level of SMA expression can be detected in NAF *in vivo* (personal observation) and when grown in 3D-culture on ECM, as presented previously.
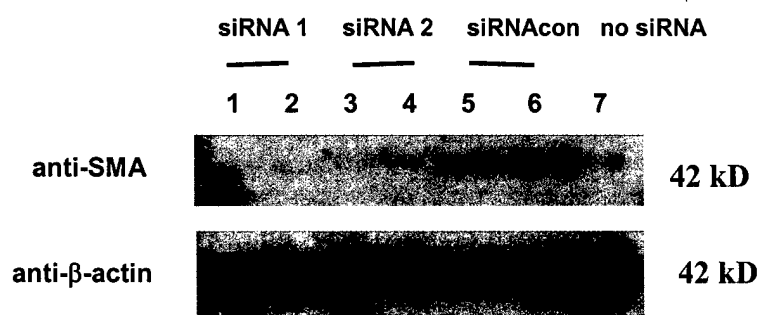

Figure 2. Transfection of siRNA specific for SMA in a single CAF culture suppressed expression of SMA (Western blot analysis). Two different double stranded siRNAs specific for SMA were used. Lanes 1 and 2 = siRNA 1, Lanes 3 and 4 = siRNA 2, Lanes 5 and 6 = a control siRNA consisting of a scrambled version of siRNA 2, Lane 7 = non-transfected CAF.
The SMA specific siRNA did not affect expression of β-actin. 10 ug of protein were loaded per lane.

We are currently assessing the duration of effect of the siRNA for SMA and whether the suppression will persist when CAF are grown in 3D on Matrigel. The preliminary results of these studies suggest that the effect of the siRNA persists on Matrigel; however, the duration of the effect is variable, but is less than one week. We have also found that our transfection protocol can have variable levels of toxicity on different CAF cultures. Therefore, we have begun utilizing the pSUPER RNAi System which provides long-term loss-of-function phenotypes in mammalian cell lines [13]. The pSUPER vector accommodates an insert that is specific for the target sequence, in our case SMA, to produce sequence specific double stranded siRNA. We will use the retroviral version of pSUPER (pSUPER.retro.puro, OligoEngine) to generate retroviral supernatants for transduction. This should provide a longer duration of suppression of SMA expression, sufficient to complete Task 2, and may be less toxic to our primary fibroblast cultures. Four pSUPER.retro vectors are being generated, each with a different SMA-specific insert and will be assessed.

c. **Collect RNA from each fibroblast culture, i.e., 10 CAF, 10 SMA-inhibited CAF and 10 NAF cultures, and perform expression analysis using cDNA microarrays (GEArray Q series Human Extracellular Matrix and Adhesion Molecule Gene Array and the GEArray Q series Human Common Cytokines Gene Array, SuperArray Biosciences Corporation)**

To determine the effect of SMA inhibition on the expression of ECM proteins and proteases by CAFs, RNA has been isolated from two successful transfection experiments of two different CAF cultures. Thus far, only RNA from the first successful transfection corresponding to the Western Blot pictured above has been analyzed using the Human ECM and Adhesion Molecule gene array (GEArray

Q series, SuperArray Bioscience Corporation). The isolated RNA was further purified using RNeasy purification columns (Qiagen) to attain a 260/280 ratio greater than 1.8. The manufacturer of these arrays recommends linear amplification of the RNA prior to labeling, therefore cDNA probes (one from SMA-siRNA transfectants and one from control-siRNA transfectants) were prepared from 0.3 ug of RNA each, by amplification and biotin-labeling (AmpoLabeling-LPR Kit, SuperArray Bioscience Corporation). The labeled probe was hybridized to the arrays as per the manufacturer's instructions and detected by chemiluminescence. The signal was recorded on radiographic film and the resulting images digitally photographed. The intensity of the signal was determined by densitometry. The expression levels were compared in the SMA-siRNA transfectants versus the control-siRNA transfectants after background subtraction and normalization to GAPDH using the GeneArray Analyzer software (SuperArray Biosciences Corporation). Only those genes in which there was a visible difference in intensity on the exposed film between the SMA-siRNA transfectants and the control transfectants were considered. The exposure duration with the highest number of visibly different intensity levels between the SMA-siRNA transfectants and the control-siRNA transfectants was used for analysis. The genes demonstrating a 2-fold or greater difference in expression are listed in Table 2. These include a variety of cell adhesion molecules, ECM proteins and ECM proteases, suggesting a significant impact of SMA expression on the overall CAF phenotype.

Genes With A Greater Expression in SMA-Inhibited or SMA-Expressing CAF				
SMA-Inhibited CAF	**Fold Difference**	**SMA-Expressing CAF**	**Fold Difference**	
Cell Adhesion Molecules		**Cell Adhesion Molecules**		
E-cadherin	>100			
CEACAM5	56			
Integrin β3/CD61	33			
Integrin α7	22.9			
Catenin β1	7.9			
Integrin α8	7.4			
CD44	4.1			
Integrin α6	2.7			
Integrin α5	2.1			
PECAM 1	2			
ECM Proteins		**ECM Proteins**		
ECM1	40.7	Laminin β1	2.7	
Type IV collagen, α2	14			
Caveolin 1	7			
Proteases		**Proteases**		
cathepsin L	5.2	Caspase 8	4.2	
MMP-10 (stromelysin 2)	3.2			
MMP-11 (stromelysin 3)	3			
MGEA5	3			

Given the known function of SMA in stopping the migration of fibroblasts as well as creating contractile force, it is not surprising that several cell adhesion and related molecules are included in this list. Inhibition of SMA expression resulted in increased expression of CD44, β-catenin, and integrins α5, α6, α7, α8, and β3, all of which mediate cell migration [10, 14]. CEACAM5, a member of the carcinoembryonic antigen-related cell adhesion molecule family, was also up-regulated in SMA-inhibited CAF. Members of this family have adhesive functions in many different cell types and have known functions in embryonic cell sorting and cell migration [15]. Platelet/endothelial cell adhesion molecule (PECAM) and E-cadherin were both also up-regulated. E-cadherin, which was not expressed at all in SMA-expressing fibroblasts, was expressed with SMA inhibition.

Alterations in some ECM molecules and proteases were also observed. There was an increased expression of collage Type IV, α1 chain, and extracellular matrix protein 1 (ECM1), a newly described

secretory glycoprotein that promotes angiogenesis [16], and decreased expression of laminin B1. Collage Type I, α1 chain, which is known to be increased in differentiated myofibroblasts, was decreased with SMA inhibition, but only 1.8 fold in this analysis. Caveolin 1 was also expressed at a higher level after SMA inhibition. Caveolin 1 is the principal structural component of caveolae which are abundantly present in differentiated cells, such as fibroblasts [17]. Caveolae participate in a wide variety of cellular events, including transcytosis, cholesterol trafficking, and the co-ordination of cell signaling events [18]. The expression of several ECM proteases was also increased in SMA-inhibited CAF. These were cathepsin L, MMP-10, MMP-11 and meningioma expressed antigen (MGEA5). MGEA5 is a hyaluronidase which is immunogenic in meningioma patients [19]. The up-regulation of ECM proteases by the inhibition of SMA was surprising given the reported importance of SMA-induced contractile forces on matrix remodeling. However, when the other known function of SMA, the immobilization of fibroblasts, is considered, the release of the SMA "brake" may initiate migration resulting in alterations in cell adhesion molecules and increases in proteases. Additionally, caspase 8 was down regulated with SMA inhibition. Activation of caspase 8 is important in receptor and non-receptor mediated apoptosis [20]. Myofibroblasts are eliminated in healing scars by apoptosis; therefore, this finding raises the possibility that expression of SMA may initiate the apoptotic process by increasing caspase 8.

We will repeat the experiment to compare the array results with and without cDNA probe amplification and after the transfected cells have been grown on Matrigel in 3D and on plastic. Our plan is to utilize retroviral pSUPER construct to achieve inhibition of SMA expression of an additional 8-9 CAF cultures. Probes prepared from RNA from CAF cultures with and without SMA inhibition and from NAF cultures will be hybridized to the indicated expression arrays. Whether or not the RNA will be amplified during probe preparation and the fibroblasts will be cultured on plastic or Matrigel will soon be determined based on the results of on-going experiments.

TASK 2 -   **To characterize the functional effect of SMA on the interactions of CAF and breast epithelial cells (Months 6 - 12)**

Currently into Month 5 of the project and no progress has been made on Task 2.

TASK 3 -   **Final data analysis and manuscript preparation (Months 10 - 12)**

Currently into Month 5 of the project and no progress has been made on Task 3.

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> Andrea Sadlonova

KEY RESEARCH ACCOMPLISHMENTS:

- Successful suppression of SMA expression in CAF using RNA interference
- Demonstration that SMA expression in CAF and NAF is affected by culture conditions. Specifically, SMA expression in CAF and NAF grown on plastic is similar, but SMA expression in CAF is higher than NAF when the cultures are grown in 3D on Matrigel, similar to *in vitro* findings.
- Demonstration that SMA in CAF affects the expression of multiple ECM proteins, cell adhesion molecules and proteases.

8

REPORTABLE OUTCOMES:

- Preparation of primary fibroblast cultures
     We plan to attempt immortalization of some of the fibroblast cultures via over-expression of telomerase in an attempt to generate cell lines.

CONCLUSIONS:

Although we are less than halfway through the project, our results thus far indicate that SMA in CAF affects the expression of several ECM proteins, cell adhesion molecules and ECM modulating proteases. This supports our hypothesis that SMA expression is important to the CAF phenotype. It also suggests that SMA may be a valid target for altering the CAF phenotype. By targeting SMA expression, could we modulate the CAF phenotype so that CAF work to inhibit the progression of breast carcinoma? To begin to answer this question we must first validate our initial results using multiple fibroblast cultures, compare the SMA-inhibited CAF phenotype with NAF, and assess the functional effect of SMA suppression on fibroblast-epithelial cell interactions. All of this work will now be completed under a recently-funded, two year grant from the National Institutes of Health (R03 CA105950-01).

We are currently working to optimize our RNA interference strategy and culture conditions. Because of the relatively short duration of effectiveness of the transfected double stranded siRNA and some difficulty with the transfection of primary fibroblasts, we are pursuing an alternative RNA interference strategy using a pSUPER retroviral construct. Furthermore, we want our culture conditions to reflect, as closely as possible, the *in vivo* phenotypes of NAF and CAF and plan to culture the fibroblasts on Matrigel prior to RNA extraction for expression array analysis. These improvements will be incorporated into our future work.

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APPENDICES:

None